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<b>(54) Title:</b> VACCINES CONTAINING ATTENUATED BACTERIA  <b>(57) Abstract</b>  The invention relates to a vaccine comprising a bacterium attenuated by a non-reverting mutation in a gene encoding a protein which promotes folding of extracytoplasmic proteins. Such mutations were initially identified as being useful in vaccines from a bank of randomly inserted, transposon mutants in which attenuation was determined as a reduction in virulence of the organism in the mouse model of infection. Site directed mutation of the gene results in a strain which shows at least 4 logs of attenuation when delivered both orally and intravenously. Animals vaccinated with such a strain are protected against subsequent challenge with the parent wild type strain. Finally, heterologous antigens such as the non-toxic and protective, binding domain from tetanus toxin, fragment C, can be delivered via the mucosal immune system using such strains of bacteria. This results in the induction of a fully protective immune response to subsequent challenge with native tetanus toxin.		

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## VACCINES CONTAINING ATTENUATED BACTERIA

5           The invention relates to vaccines containing attenuated bacteria.

### Background to the invention

          The principle behind vaccination is to induce an immune response in the host  
10   thus providing protection against subsequent challenge with a pathogen. This may be  
      achieved by inoculation with a live attenuated strain of the pathogen (i.e. a strain  
      having reduced virulence such that it does not cause the disease caused by the  
      virulent pathogen).

15       Classically, live attenuated vaccine strains of bacteria and viruses have been  
      selected using one of two different methodologies. Mutants have been created either  
      by treatment of the organism using mutagenic chemical compounds or by repeated  
      passage of the organism *in vitro*. However, use of either method gives rise to  
      attenuated strains in which the mode of attenuation is unclear. These strains are  
20   particularly difficult to characterize in terms of possible reversion to the wild type  
      strain as attenuation may reflect single (easily reversible) or multiple mutation  
      events.

          Using modern genetic techniques, it is now possible to construct genetically  
25   defined attenuated bacterial strains in which stable attenuating deletions can be  
      created. A number of site directed mutants of *Salmonella* have been created using  
      this type of technology (2, 5, 6, 12, 22, 35, 36, 37). Amongst the most  
      comprehensively studied attenuating lesions are those in which mutations in the  
      biosynthetic pathways have been created, rendering the bacteria auxotrophic (e.g. *aro*  
30   genes). Mutations in these genes were described as early as 1950 (1) as responsible  
      for rendering *Salmonella* less virulent for mice. Several different auxotrophic  
      mutations such as *galE*, *aroA* or *purA* have also been described previously (6, 12).  
      *Salmonella aroA* mutants have now been well characterised and have been shown to

be excellent live vaccines against salmonellosis in several animal species. In addition, in order to reduce the chances of a reversion to virulence by a recombination event mutations have now been introduced into two independent genes such as *aroA/purA* and *aroA/aroC*. Identical mutations in host adapted strains of *Salmonella* such as *S. typhi* (man) and *S. dublin* (cattle) has also resulted in the creation of a number of single dose vaccines which have proved successful in clinical (11, 17) and field trials (15).

In animal studies, attenuated *S. typhimurium* has been used as a vehicle for the delivery of heterologous antigens to the immune system (3, 8, 32). This raises the potential of the development of multivalent vaccines for use in man (9).

#### Summary of the invention

The original aim of the work that led to the invention was the identification of novel genes that are involved in the virulence pathways of pathogenic bacteria, the identification and deletion of which may render the bacteria avirulent and suitable for use as vaccines. To identify attenuating lesions, random mutations were introduced into the chromosome of *S. typhimurium* using the transposon *TnphoA* (18). This transposon is unique in that it is engineered to identify proteins that are expressed in or at the bacterial outer membrane; such proteins may be those involved in interaction with and uptake by host tissues. By using the natural oral route of infection to screen these mutants, those with important, *in vivo* induced, attenuating lesions in genes were identified.

25

One such gene identified through this work is *surA*. The *surA* gene product is known to promote the folding of extracytoplasmic proteins. Accordingly, the invention provides a vaccine comprising a pharmaceutically acceptable carrier or diluent and a bacterium attenuated by a non-reverting mutation in a gene encoding a protein which promotes the folding of extracytoplasmic proteins. The vaccine has the ability to confer protection against a homologous wild type oral challenge with

the virulent bacterium. In addition, the bacterium used in the vaccine can act as a carrier for heterologous antigens such as fragment C of tetanus toxin.

### Detailed description of the invention

5

#### **Proteins that promote the folding of extracytoplasmic proteins**

Periplasmic and outer membrane proteins are secreted across the cytoplasmic (inner) membrane in a mostly unfolded state, and they then fold after secretion. The folding often has enzymatic assistance to catalyse the formation of bonds necessary for the protein to reach its folded state. For example, the folding often requires the participation of enzymes that catalyse the formation of disulphide bonds or enzymes that catalyse the isomerisation of prolyl bonds (peptidyl-prolyl cis-trans isomerases or PPIases).

15

One known PPIase is SurA. The inventors have now shown that mutation of the *surA* gene causes attenuation of virulent bacteria and that the attenuated bacteria are useful as vaccines.

20 SurA was first described as being essential for the survival of *E.coli* in the stationary phase (33). It is a periplasmic protein. More recently, SurA has been described as belonging to a third, new family of PPIases (30), the parvulin family. Further studies have shown SurA to be involved in the correct folding of outer membrane proteins such as OmpA, OmpF, and LamB (16, 24, 29).

25

PPIases are divided into three families, the cyclophilins, FK506-binding proteins (FKBPs) and parvulins. Members of all three families have been found in *E.coli*. Apart from SurA, the parvulin family includes several proteins such as NifM, PrsA and PrtM.

30

### Bacteria useful in the invention

The bacteria that are used to make the vaccines of the invention are generally those that infect via the oral route. The bacteria may be those that invade and grow  
5 within eukaryotic cells and/or colonise mucosal surfaces. The bacteria are generally Gram-negative.

The bacteria may be from the genera *Salmonella*, *Escherichia*, *Vibrio*, *Haemophilus*, *Neisseria*, *Yersinia*, *Bordetella* or *Brucella*. Examples of such bacteria  
10 are *Salmonella typhimurium* – the cause of salmonellosis in several animal species; *Salmonella typhi* – the cause of human typhoid; *Salmonella enteritidis* – a cause of food poisoning in humans; *Salmonella choleraesuis* – a cause of salmonellosis in pigs; *Salmonella dublin* – a cause of both a systemic and diarrhoeal disease in cattle, especially of new-born calves; *Escherichia coli* – a cause of diarrhoea and food  
15 poisoning in humans; *Haemophilus influenzae* – a cause of meningitis; *Neisseria gonorrhoeae* – a cause of gonorrhoeae; *Yersinia enterocolitica* – the cause of a spectrum of diseases in humans ranging from gastroenteritis to fatal septicemic disease; *Bordetella pertussis* – the cause of whooping cough; or *Brucella abortus* – a cause of abortion and infertility in cattle and a condition known as undulant fever in  
20 humans.

*Salmonella* bacteria are particularly useful in the invention. As well as being vaccines in their own right against infection by *Salmonella*, attenuated *Salmonella* can be used as carriers of heterologous antigens from other organisms to the immune  
25 system via the oral route. *Salmonella* are potent immunogens and are able to stimulate systemic and local cellular and antibody responses. Systems for driving expression of heterologous antigens in *Salmonella in vivo* are known; for example the *nirB* and *htrA* promoters are known to be effective drivers of antigen expression *in vivo*.

30

The invention is also particularly applicable to *E.coli*, especially

enterotoxigenic *E. coli* ("ETEC"). ETEC is a class of *E. coli* that cause diarrhoea. They colonise the proximal small intestine. A standard ETEC strain is ATCC H10407.

5        Infections of ETEC are the single most frequent cause of travellers diarrhoea, causing 3-9 million cases per year amongst visitors to developing countries. In endemic areas, ETEC infections are an important cause of dehydrating diarrhoea in infants and young children, resulting in 800,000 deaths a year in the under fives world-wide. In developing countries, the incidence of ETEC infections leading to  
10 clinical disease decreases with age, indicating that immunity to ETEC infection can be acquired. In contrast, naive adults from industrialized countries who visit endemic areas are highly susceptible to ETEC infections. However, with prolonged or repeated visits to endemic areas susceptibility to ETEC infections diminishes, suggesting that a live attenuated approach to ETEC vaccination may prove  
15 successful.

Seq. Id. No. 1 shows the sequence of the *surA* open reading frame in *Salmonella typhimurium*, and Seq. Id. No. 2 shows the sequence of the *surA* open reading frame in *E. coli*.

20

#### Second mutations

The bacteria used in vaccines of the invention preferably contain a mutation in one or more genes in addition to the mutation in the gene encoding a protein which  
25 promotes folding of extracytoplasmic proteins. This is so that the risk of the bacterium reverting to the virulent state is minimised, which is clearly important for the use of the bacterium as a human or animal vaccine. Although bacteria containing only a mutation in a protein which promotes folding of extracytoplasmic proteins are attenuated and the risk of reversion is small, it will generally be desirable to introduce  
30 at least one further mutation so as to reduce the risk of attenuation yet further.

A number of genes that are candidates for second and further mutations are known (see e.g. ref 39). These include the *aro* genes (35), the *pur* genes, the *htrA* gene (37), the *ompR* gene (36), the *galE* gene, the *cya* gene, the *crp* gene or the *phoP* gene. The *aro* gene may be *aroA*, *aroC*, *aroD* or *aroE*. The *pur* gene may be *purA*,  
5 *purB*, *purE* or *purH*. The use of *aro* mutants, especially double *aro* mutants, is preferred because such mutants have been shown to be particularly effective as vaccines. Suitable combinations of *aro* mutations are *aroAaroC*, *aroAaroD* and *aroAaroE*.

## 10 The nature of the mutation

The mutations introduced into the bacterial vaccine generally knock-out the function of the gene completely. This may be achieved either by abolishing synthesis of any polypeptide at all from the gene or by making a mutation that results in  
15 synthesis on non-functional polypeptide. In order to abolish synthesis of any polypeptide, either the entire gene or its 5'-end may be deleted. A deletion or insertion within the coding sequence of a gene may be used to create a gene that synthesises only non-functional polypeptide (e.g. polypeptide that contains only the N-terminal sequence of the wild-type protein). In the case of mutations in genes  
20 encoding proteins which promote the folding of extracytoplasmic proteins, the mutation generally abolishes the ability of the protein to promote such protein folding.

The mutations are non-reverting mutations. These are mutations that show  
25 essentially no reversion back to the wild-type when the bacterium is used as a vaccine. Such mutations include insertions and deletions. Insertions and deletions are preferably large, typically at least 10 nucleotides in length, for example from 10 to 600 nucleotides.

30 The bacterium used in the vaccine preferably contains only defined mutations, i.e. mutations which are characterised. It is clearly undesirable to use a bacterium



which has uncharacterised mutations in its genome as a vaccine because there would be a risk that the uncharacterised mutations may confer properties on the bacterium that cause undesirable side-effects.

5       The attenuating mutations may be constructed by methods well known to those skilled in the art (see ref 31). One means for introducing non-reverting mutations into extracytoplasmic proteins is to use transposon *TnphoA*. This can be introduced into bacteria to generate enzymatically active protein fusions of alkaline phosphatase to extracytoplasmic proteins. The *TnphoA* transposon carries a gene  
10 encoding kanamycin resistance. Transductants are selected that are kanamycin resistant by growing colonies on an appropriate selection medium.

Alternative methods include cloning the DNA sequence of the wild-type gene into a vector, e.g. a plasmid or cosmid, and inserting a selectable marker into the  
15 cloned DNA sequence or deleting a part of the DNA sequence, resulting in its inactivation. A deletion may be introduced by, for example, cutting the DNA sequence using restriction enzymes that cut at two points in the coding sequence and ligating together the two ends in the remaining sequence. A plasmid carrying the inactivated DNA sequence can be transformed into the bacterium by known  
20 techniques. It is then possible by suitable selection to identify a mutant wherein the inactivated DNA sequence has recombined into the chromosome of the bacterium and the wild-type DNA sequence has been rendered non-functional in a process known as homologous recombination.

## 25 Expression of heterologous antigens

The attenuated bacterium used in the vaccine of the invention may be genetically engineered to express an antigen from another organism (a "heterologous antigen"), so that the attenuated bacterium acts as a carrier of the antigen from the  
30 other organism. In this way it is possible to create a vaccine which provides protection against the other organism. A multivalent vaccine may be produced which

not only provides immunity against the virulent parent of the attenuated bacterium but also provides immunity against the other organism. Furthermore, the attenuated bacterium may be engineered to express more than one heterologous antigen, in which case the heterologous antigens may be from the same or different organisms.

5

The heterologous antigen may be a complete protein or a part of a protein containing an epitope. The antigen may be from another bacterium, a virus, a yeast or a fungus. More especially, the antigenic sequence may be from tetanus, hepatitis A, B or C virus, human rhinovirus such as type 2 or type 14, herpes simplex virus, poliovirus type 2 or 3, foot-and-mouth disease virus, influenza virus, coxsackie virus or *Chlamydia trachomatis*. Useful antigens include *E.coli* heat labile toxin B subunit (LT-B), *E.coli* K88 antigens, P.69 protein from *B. pertussis* and tetanus toxin fragment C.

15 The DNA encoding the heterologous antigen is expressed from a promoter that is active *in vivo*. Two good promoters are the *nirB* promoter (38, 40) and the *htrA* promoter (40).

A DNA construct comprising the promoter operably linked to DNA encoding the heterologous antigen may be made and transformed into the attenuated bacterium using conventional techniques. Transformants containing the DNA construct may be selected, for example by screening for a selectable marker on the construct. Bacteria containing the construct may be grown *in vitro* before being formulated for administration to the host for vaccination purposes.

25

#### Formulation of the vaccine

The vaccine may be formulated using known techniques for formulating attenuated bacterial vaccines. The vaccine is advantageously presented for oral administration, for example in a lyophilised encapsulated form. Such capsules may be provided with an enteric coating comprising, for example, Eudragate "S" (Trade

Mark), Eudragate "L" (Trade Mark), cellulose acetate, cellulose phthalate or hydroxypropylmethyl cellulose. These capsules may be used as such, or alternatively, the lyophilised material may be reconstituted prior to administration, e.g. as a suspension. Reconstitution is advantageously effected in a buffer at a suitable pH to ensure the viability of the bacteria. In order to protect the attenuated bacteria and the vaccine from gastric acidity, a sodium bicarbonate preparation is advantageously administered before each administration of the vaccine. Alternatively, the vaccine may be prepared for parenteral administration, intranasal administration or intramuscular administration.

10

The vaccine may be used in the vaccination of a host, particularly a human host but also an animal host. An infection caused by a microorganism, especially a pathogen, may therefore be prevented by administering an effective dose of a vaccine prepared according to the invention. The dosage employed will be dependent on various factors including the size and weight of the host and the type of vaccine formulated. However, a dosage comprising the oral administration of from  $10^7$  to  $10^{11}$  bacteria per dose may be convenient for a 70kg adult human host.

## 20 Examples

The following Examples serve to illustrate the invention.

### **Brief description of the drawings**

25

Figure 1: Southern blot confirming the defined deletion created within *surA* in the strain BRD 1115. Lanes 1 and 2 have been restricted using the enzyme *Pst*I, lanes 3-10 have been restricted with *Sal*I. The filters have been probed using a 500 bp PCR product that contains a 500 bp fragment from the middle of the *surA* gene. Lanes 2 and 4 show hybridisation of this probe to a band 500 bp smaller than the corresponding wild type lanes 1 and 3. The transposon mutant BRD441 shows

hybridisation to 2 bands since the enzyme *SaII* cuts the transposon into two. HB101 shows no hybridisation whilst the other wild type *Salmonella* strains show the same hybridisation as C5 when restricted with *SaII*.

5 Figure 2: This figure shows the colonisation and persistence of BRD1115, BRD441 and the wild type C5 in the mesenteric lymph nodes (top left graph), Peyer's patches (bottom right), spleens (bottom left) and livers (top right) in BALB/c mice following oral inoculation. The x-axis is time in days and the y-axis is  $\log_{10}$  CFU/ml (CFU stands for colony forming units).

10

Figure 3: Three strains were constructed to evaluate the ability of mutant *Salmonella* strains to deliver the heterologous antigen Fragment C in the mouse. BRD1115 is the parental strain. Two plasmids encoding the Fragment C gene of tetanus toxin under the control of either the *htrA* or *nirB* promoter were introduced into the strain

15 BRD1115 to give the strains BRD1127 and 1126 respectively. Expression of fragment C was determined *in vitro* by Western blotting. These strains were then used in an *in vivo* experiment using BALB/c mice. Groups of 10 mice were immunised orally with  $\log_{10}8$  organisms each of the 3 strains. Serum samples were taken weekly and analysed for total antibodies against tetanus toxin fragment C. The  
20 titres of anti-fragment C were determined as the highest sample dilution giving an absorbance value of 0.3 above normal mouse serum. The highest sample dilution tested was 1/6250. All mice immunised with BRD 1126 showed antibody titres higher than 6250.

25 Figure 4: Schematic showing a plasmid map of pLG339/*surA*.

Figure 5: Graph showing the survival of Balb/c mice following oral challenge with  $\log_{10}8$  bacteria of the three strains C5, BRD1115 and K2.

30

### Example 1

This Example shows the identification of mutations in *surA* as attenuating mutations, the construction of a defined *surA* mutation and the evaluation of a *surA* mutant as a vaccine (both against homologous challenge and as a carrier for heterologous antigens).

### **Materials and methods**

#### 10 1.1 Bacteria, bacteriophage, plasmids and growth conditions

The bacteria used in this study are listed in Table 1. Bacteria were routinely cultured on L-agar or in L-broth containing 100µg/ml ampicillin or 50µg/ml kanamycin where appropriate. The bacteriophage P22HT105/1int is a high frequency transducing bacteriophage obtained from Dr Tim Foster (Trinity College, 15 Dublin). The plasmid pGEM-T (Promega Corporation, USA) is designed for direct cloning of PCR fragments and pBluescript<sup>0</sup>II SK+ (Stratagene Ltd, Cambridge, U.K.) is a general cloning vector. The other plasmids are described in the text.

#### 1.2 Purification of DNA and DNA manipulation techniques

20 All DNA manipulation including Southern blotting were carried out as described by Sambrook *et al* (31). Restriction enzymes and T4 DNA ligase were purchased from Boehringer Mannheim (Lewes, UK) and used according to the manufacturers instructions. Chromosomal DNA preparation was prepared according to the method of Hull (13).

25

#### 1.3 DNA sequencing

Double stranded plasmid sequencing was carried out using the Sequenase kit (Trade Mark, United States Biochemical Corporation) according to the manufacturers' instructions. Labelling of the DNA was achieved using <sup>35</sup>S-dATP 30 (Amersham, UK) and fragments separated on an 8% acrylamide/bis-acrylamide gel containing 7M urea, for 2hours at 35 mA.

#### 1.4 DNA amplification by polymerase chain reaction

Polymerase chain reactions (PCR) were carried out with *Taq* DNA polymerase using the GeneAmp kit (Trade Mark, Perkin Elmer Cetus, USA) according to the manufacturers' instructions. Oligonucleotides were purchased from the Molecular Medicine Unit, Kings College, London and the sequences are shown in Table 1. Mixtures of DNA and specific primers were subjected to multiple rounds of denaturation, annealing and extension in the presence of the enzyme *Taq* polymerase. 100 ng plasmid DNA and 1mg chromosomal DNA were added to a mixture containing 5µl 10 x buffer (100mM Tris-HCl, pH 8.3; 500mM KCl; 15mM Mg Cl<sub>2</sub>; 0.01% gelatine(v/v)); 8µl of deoxy-nucleotide mixture (1.25mM each of deoxy-nucleotide triphosphate; dATP, dCTP, dGTP and dTTP); 1µl of a 10µM sense primer; 1µl of a 10µM anti-sense primer and 2.5 units *Taq* polymerase. This mixture is overlaid with 50µl light mineral oil (Sigma) to prevent evaporation and the tubes incubated in an Omnigene Thermal Cycler (Trade Mark, Hybaid). Amplification of the DNA was performed using the following programme: 1 cycle of 95°C for 5 minutes, 50°C for 1.5 minutes, 74°C for 2 minutes; 19 cycles of 95°C for 1.5 minutes, 50°C for 2 minutes, 74°C for 3 minutes; 10 cycles of 95°C 2 minutes, 50°C for 2 minutes, 74°C for 7 minutes.

#### 1.5 Transformation of bacteria

##### 1.5.1. Heat shock

Bacteria are rendered competent to DNA uptake by the calcium chloride method. An overnight bacterial culture was used to seed a fresh 25 ml LB broth culture (a 1:100 dilution) which was grown aerobically with shaking until the cells reached mid-log growth phase (OD 650nm = 0.4 to 0.6). The cells were harvested by centrifugation at 3000 x g for 10 minutes at 4°C. The supernatant was discarded and the pellet resuspended in 25 ml ice-cold 75mM CaCl<sub>2</sub>. The process was repeated and the cells incubated on ice for 30 minutes. The cells were pelleted by centrifugation at 3000 x g for 10 minutes at 4°C. The cell pellet was resuspended in 1.2 ml ice-cold 75mM CaCl<sub>2</sub> and stored on ice until needed. The cells were then competent to DNA uptake. A maximum of 20µl of the ligation mix was added to 200µl of the

competent cells and the mixture stored on ice for 30 minutes. The cells were then subjected to heat shock by incubation in a 42°C waterbath for 2 minutes. The cells were then transferred back to ice for a further 2 minutes. 1 ml of LB broth was added to the mixture and the cells incubated at 37°C for at least 60 minutes to allow  
5 expression of the antibiotic marker on the plasmid. 100µl aliquots of cells were plated onto LB agar plates containing the appropriate antibiotics and incubated overnight at 37°C.

#### 1.5.2. Electroporation

10 Plasmid DNA was introduced into bacterial strains using electroporation. Mid-log phase growth cultures were generated as for the heat-shock method and the cells pelleted by centrifugation at 3000 x g for 10 minutes at 4°C. The cell pellet was washed twice with an equal volume of ice-cold 10% glycerol and pelleted as before. The cell pellet was resuspended in 300-500µl ice-cold 10% glycerol. Approximately  
15 100 ng plasmid (or 1 µg suicide vector) in a volume not greater than 6 µl sterile water was added to 60 µl competent cells in a pre-chilled electroporation cuvette on ice. The plasmid was electroporated into the bacteria using a Bio-Rad Gene Pulser (Trade Mark) with the following conditions 1.75kV, 600Ω, 25µF. 1ml LB broth was then added to the contents of the electroporation cuvette and the mixture incubated at 37°C  
20 for 90 minutes to allow the cells to recover. 100 µl aliquots of the electroporation mix were plated out onto selection media and incubated at 37°C overnight.

#### 1.6 P22 Transduction

Transduction experiments were carried out using the bacteriophage P22  
25 HT105/1 int<sup>-</sup>. Phage lysates were prepared using LB5010 as the donor strain. A 5ml overnight culture of LB5010 was grown in L broth containing 0.2% glucose and galactose to increase the expression of phage receptors on the cell surface. Ten fold serial dilutions of the P22 stock were made in TMGS up to 10<sup>-8</sup> (stock is approximately 10<sup>10</sup> pfu/ml). 10µl of each dilution was added to 100µl of the  
30 overnight stock of cells and incubated at 37°C for 30-45 minutes to allow adsorption of the phage to the cells. 3mls of top agar was added to each incubation and spread

onto L agar plates containing 100µg/ml ampicillin. The plates were incubated at 37°C for approximately 4-5 hours until plaques were visible. The dilution that gave almost confluent plaques after this length of time was the one chosen for harvesting. The plaques were harvested by scraping the top agar into 2ml of phage buffer with a glass microscope slide. A few drops of chloroform were added and the phage stock stored at 4°C until needed. The recipient strain C5 was grown during the day in L broth at 37°C until late log/stationary phase. 1µl, 5µl, 10µl, 20µl, and 50µl aliquots of the new phage stock were added to 100µl aliquots of the recipient strain and incubated at 37°C for 1 hour. The cells were then spread onto L agar ampicillin plates containing 5mM EGTA (to prevent phage replication) and incubated at 37°C overnight. Colonies were replated onto L agar ampicillin plates containing 5mM EGTA three times to ensure that they were free from phage. The colonies no longer had a jagged appearance thus indicating an absence of phage.

#### 15 1.7 *In vitro* analysis of bacterial strain

##### 1.7.1. Agglutination with antisera

Agglutination using anti-sera raised against the O antigen of *Salmonella* can be used as a rapid test not only for the integrity of the bacterial LPS but also as a diagnostic of the strain, e.g. anti-sera against the 04 and 05 antigens for *S.typhimurium*. These were obtained from Murex Diagnostics Ltd (Dartford U.K.). A sweep of colonies was harvested from the growth on a plate incubated overnight, and resuspended in 100µl PBS. This sample was mixed with a drop of antisera on a glass slide and the agglutination compared with a positive and negative sample.

##### 25 1.7.2 HEp-2 invasion assay

The HEp-2 cell line is an adherent epidermoid carcinoma derived from human larynx (ATCC CCL23). It can be cultured as a monolayer in Dulbecco's modified Eagle's medium with 10% FCS, glutamine and penicillin/streptomycin at 37°C in the presence of 5% CO<sub>2</sub>. Confluent cells were detached from the tissue culture flasks by the use of trypsin/EDTA. The cells were first washed in PBS to remove any serum that might affect the action of the trypsin. Trypsin/EDTA was then added to the



monolayer and the cells incubated at 37°C for 5 minutes. The cells were removed from the plastic by gentle tapping on the edge of the flask. The trypsin was neutralised with 1.5 volumes of DMEM. Cells are collected by centrifugation at 1000 x g for 5 minutes. The supernatant was removed and the cell pellet resuspended in DMEM. The cell pellet was counted and the concentration adjusted to give  $2 \times 10^5$  cells per ml.

1 ml of the cell suspension was added to one well of a 24 well tissue culture plate (Costar 3524), three wells for each bacterial strain being investigated. The cells were incubated overnight to form a confluent monolayer in the well. The cells were then washed 5 times with PBS to ensure removal of the antibiotics and 1 ml DMEM added (without any antibiotics).  $1 \times 10^7$  bacteria were added to each well and incubated at 37°C for 3 hours. The cells were washed 3 times with PBS to remove any extracellular bacteria. 1 ml of DMEM containing 100 µg/m gentamycin was added and the cells incubated for a further 1 hour. The cells were washed 5 times with PBS. The cells were lysed by the addition of 1 ml of 0.1% Triton-X-100 at 37°C for 15 minutes. The cells were further lysed by agitation with a blue pipette tip and the lysate transferred to a 1.5 ml centrifuge tube. The viable bacteria that had invaded the cells were counted using the Miles-Misra drop test method (19).

20

### 1.8. *In vivo* analysis of bacterial strains

#### 1.8.1. Preparation of live bacteria for immunisation of mice.

A vial of the appropriate strain was thawed from liquid nitrogen and used to inoculate a 250 ml culture of LB broth containing antibiotic where appropriate. The culture was grown overnight at 37°C without shaking. The bacteria were harvested by centrifugation at 3000 x g for 10 minutes and washed once in sterile PBS. The bacteria were harvested again by centrifugation and resuspended in 5 ml sterile PBS. The concentration of bacteria was estimated by optical density at 650 nm using a standard growth curve for that strain. Based on this estimate the cell concentration was adjusted with PBS to that required for immunisation. A viable count was

30

prepared of each inoculum to give an accurate number of colony forming units per ml (cfu/ml) administered to each animal.

#### 1.8.2. Oral immunisation of mice with live bacteria.

- 5        The mice were lightly anaesthetised with a mixture of halothane and oxygen and the bacteria administered by gavage in 0.2 ml volumes using a gavage needle attached to a 1ml syringe.

#### 1.8.3. Intravenous (i.v.) immunisation of mice with live bacteria.

- 10       Mice were placed in a warm chamber and 0.2 ml volumes injected into a tail vein of each mouse using a 27 gauge needle.

#### 1.8.4. Enumeration of viable bacteria in mouse organs.

- Groups of four or five mice were sacrificed up to 7 weeks post oral  
15 immunisation with three bacterial strains. Spleens, livers, mesenteric lymph nodes and Peyer's patches were removed and homogenised in 10ml sterile PBS using a stomacher (Colworth, U.K.). Dilutions of these homogenates were plated out in LB agar with kanamycin if required and incubated overnight at 37°C. The number of viable bacteria present in each homogenate was then calculated from the dilution.

20

#### 1.9. Determination of antibody titres against fragment C.

- Serum antibody responses against fragment C were measured by enzyme-linked immunosorbant assay (ELISA) as previously described (28) using 96 well EIA/RIA plates (Costar 3590). Absorbance values were read at A<sub>490</sub> and plotted  
25 against dilutions (data not shown). A normal mouse serum control was added to each ELISA plate and used to define the background level response.

#### 1.10 Tetanus toxin challenge

- Mice were challenged with 0.05 µg (50 x 50% lethal doses) of purified  
30 tetanus toxin as previously described (7), and fatalities recorded for 4 days.

## Results

### 2.1 Cloning and mapping of *TnphoA* insertion sites

A number of *S.typhimurium* *TnphoA* insertion mutants were previously identified as being attenuated when administered orally to BALB/c mice. In addition some of these mutants also exhibited a reduced ability to invade the cultured epithelial cell line HEp-2. To identify the genes that had been disrupted by the *TnphoA* insertion, genomic DNA was digested using *Sau3A* and cosmid banks prepared from each strain. These banks were screened using *TnphoA* probes and cosmid exhibiting homology with the 3' and 5' probes were examined. Fragments from these cosmid were cloned into the vector pBluescript<sup>®</sup> II SK+. The nucleotide sequence surrounding these insertion sites was determined and the genes identified. Two insertions were found to be within the *htrA* gene (14), one in the *osmZ* gene (10) and one in the *surA* gene.

15

The *surA* gene open reading frame of *Salmonella typhimurium* shown in Seq Id No. 1 is 1281 bases long, encoding a protein of some 427 amino acids with a molecular weight of 47.2Kd. This protein is virtually identical to that found in *E.coli* (34), and is described as being essential for survival in long term culture (33). The *surA* gene contains a leader peptidase cleavage site indicating that this is a transported protein. It has now been described as belonging to a peptidyl prolyl isomerase family, with a function to aid the correct folding of outer membrane proteins (16, 24, 29).

### 2.2 Introduction of a defined deletion into the *surA* gene.

Restriction analysis and DNA sequencing of the *surA* gene revealed the presence of single *HpaI* and *SmaI* restriction enzyme sites within the coding region of the gene which could be used to generate a deletion of 400 bases. The plasmid pGEM-T/212/213 was constructed containing a 3Kb region encompassing the entire *surA* gene and flanking region. Digestion of the plasmid with the enzymes *HpaI* and *SmaI*, gel purification of the large 5.5Kb fragment and re-ligation resulted in a

plasmid containing a 419bp deletion within the *surA* gene. This plasmid was designated pGEM-T/ $\Delta$ surA.

### 2.3 Introduction of the *surA* deletion into the chromosome of *S.typhimurium* C5.

5        The plasmid pGEM-T was digested with the two restriction enzymes *SphI* and *SalI*. The 2.6kb fragment containing the deleted *surA* gene was gel purified and ligated into the suicide replicon pGP704 that had previously been digested with the same enzymes. The suicide replicon pGP704 has been used previously to introduce deletions into the chromosome of *S.typhi* (4) and *S.typhimurium* (26) which lack the  
10 *pir* gene, the product of which is essential for the replication of pGP704. The ligation mix was used to transform the strain SY327, an *E.coli* strain that contains the *pir* gene, and a plasmid of the expected size identified by restriction analysis. This plasmid was designated pGP704/ $\Delta$ surA. Since suicide replicons cannot replicate in *S.typhimurium* the drug resistance marker is only expressed if there has been a single  
15 homologous recombination event, incorporating the plasmid into the bacterial chromosome.

The plasmid pGP704/ $\Delta$ surA was used to transform the semi-rough *S.typhimurium* strain LB5010 by the calcium chloride method. Three transformants  
20 were selected on agar containing ampicillin. These single crossovers were moved from this intermediate strain into the wild type C5 using P22 transduction (20). P22 lysates were prepared from the three transductants and introduced into C5. One ampicillin resistant colony was obtained from this process. This transformant was sub-cultured twice into L-broth containing no selection and grown for 48 hours.  
25 Serial dilutions of this culture were made and the  $10^{-6}$  dilution was spread onto L-agar plates containing no selection. 500 colonies were streaked by hand on to duplicate plates, one containing agar, the other agar with ampicillin. One colony was found to be ampicillin sensitive indicating the loss of the drug resistance marker of the plasmid following a second homologous recombination event.

30

This potential *surA* mutant was confirmed as a *S.typhimurium* strain by

agglutination with 04 and 05 antiserum. The deletion was confirmed by PCR using the primers MGR92 and MGR93, giving a 1 kb product. The deletion was also confirmed cloning the PCR product into the vector pGEM-T to give the plasmid pGEM-T/92/93, and sequencing across the deletion using the primers MGR130 and

5 135. Figure 1 shows the results of probing *Pst*I and *Sa*I digested genomic DNA from C5 and the *surA* mutant strain with a PCR product obtained from the wild type C5. The band seen in the *surA* mutant track is approximately 400 bases smaller than that seen in the wild type. This deleted strain was designated BRD1115.

## 10 2.4 Characterisation of the strain BRD1115

### 2.4.1 *In vitro* analysis of the invasion of cultured epithelial cells

The strain BRD1115 was tested for its ability to invade the cultured epithelial cell line HEp-2. The levels of invasion were found to be reduced by 80% in comparison to the wild type strain C5. The transposon mutant BRD441 showed a

15 90% reduction in invasion compared to C5.

### 2.4.2. Evaluation of the *in vivo* properties of BRD1115 in BALB/c mice.

#### 2.4.2.1. Determination of oral and i.v. LD<sub>50</sub>'s

The oral and i.v. LD<sub>50</sub>'s of BRD 1115, C5 and BRD441 were calculated

20 using the mouse susceptible strain BALB/c. 5 mice per group were inoculated either orally or i.v. with doses ranging from log<sub>10</sub> 4 to log<sub>10</sub> 10 orally and log<sub>10</sub> 1 to log<sub>10</sub> 5 i.v. Deaths were recorded over 28 days and the LD<sub>50</sub>'s calculated by the method of Reed and Meunch (27). BRD1115 was determined to show nearly 5 logs of attenuation orally and 3.5 logs i.v compared to C5. BRD441 showed 4.5 logs attenuation orally

25 and 1 log i.v.. The results are presented in Table 2.

#### 2.4.2.2. Persistence of strains in the organs of BALB/c mice following oral inoculation

Groups of 4 BALB/c mice were orally inoculated with log<sub>10</sub> 8 organisms of the

30 three strains. Mice were killed at days 0,1,4,7,10,16,21 and 28 and the organs examined for bacterial load. The wild type strain C5 colonised the spleen, liver,

mesenteric lymph nodes and Peyer's patches in high numbers ( $>\log_{10}4$  cfu/ml), eventually resulting in the death of the animals. BRD 1115 and BRD 441 on the other hand persisted in the liver and spleens for more than 40 days in low numbers ( $<\log_{10}2$  cfu/ml). These results are presented in Figure 2.

5

## 2.5. Evaluation of BRD1115 as a potential vaccine strain

### 2.5.1. BRD1115 protects against homologous challenge

Groups of BALB/c mice were orally immunised with  $\log_{10}8$  organisms of BRD1115 and challenged with the wild type strain C5 at 4 weeks and 10 weeks post inoculation. The mice were challenged with  $\log_{10}4$  to  $\log_{10}10$  organisms C5 and a new oral  $LD_{50}$  calculated. The levels of protection are presented in Table 3, showing  $\log_{10}4$  protection after 4 weeks and  $\log_{10}5$  after 10 weeks.

### 2.5.2. BRD1115 as a potential carrier strain for heterologous antigens

15 Two plasmids encoding the C fragment of tetanus toxin were introduced into two isolates of BRD1115 by electroporation. The plasmids are pTET*nir*15 (38) in which fragment C is under the control of the *nirB* promoter, and pTET*htrA* in which fragment C is under the control of the *htrA* promoter. The plasmids were found to be maintained at levels greater than 90% in BRD1115 even when the selection pressure  
20 of ampicillin was removed from the growth medium. *In vitro* expression of fragment C was determined by Western blotting. The strains were cultured under both inducing (42°C for BRD 1126 and anaerobiosis for BRD 1127) and non-inducing conditions (37°C for BRD 1126 and aerobiosis for BRD 1127). A higher level of expression was seen for both strains under inducing conditions with BRD 1127  
25 showing higher levels of fragment C expression than BRD 1126.

Groups of 10 BALB/c mice were orally immunised with  $\log_{10}8$  organisms and bled weekly. The titres of anti-fragment C antibodies present in the serum of each animal was determined by ELISA. The titres were determined as the reciprocal of  
30 the highest sample dilution giving an absorbance of 0.3 above normal mouse serum. The results are presented in Figure 3.

Four weeks post immunisation the mice were challenged with 50LD<sub>50</sub>'s of tetanus toxin subcutaneously and the deaths noted over 4 days. The results are presented in Table 4, showing that 100% protection was given after immunisation with BRD1127  
5 (fragment C under the control of the *htrA* promoter) and 60% protection after immunisation with BRD1126 (under *nirB* promoter). No naive mice survived the challenge.

### Example 2

10

This Example confirms that the mutation in *surA* is responsible for the attenuation. This was determined by complementation of the deleted gene with an intact version of the gene expressed on a plasmid. The complemented strain was as virulent as the wild-type organism given orally to mice.

15

### **Materials and Methods**

#### 3.1 Construction of plasmid containing the intact *surA* gene

pLG339 (41) is a low copy number plasmid based on pSC105. A 3kb  
20 fragment of the plasmid pGEM-T/212/213 (section 2.2) containing the intact *surA* gene and flanking region was cloned into the *SphI/SalI* sites of the plasmid pLG339 to create the plasmid pLG339/*surA*. A schematic of this plasmid is shown in Figure 4.

#### 25 3.2 Introduction of the plasmid pLG339/*surA* into defined mutant strain BRD1115

The plasmid was electroporated into electrocompetent BRD 1115 as previously described in section 1.5.2. Transformants containing the plasmid were selected by plating the electroporation mix onto agar plates containing 15µg/ml kanamycin. Plasmid DNA was recovered from a single colony of this transformation  
30 and checked for identity by restriction analysis. This strain was called K2.

### 3.3 Plasmid stability within the strain K2.

The ability of the intact *surA* gene on the plasmid to complement the action of the deleted *surA* gene in the chromosome relies on the plasmid being retained within the bacterial strain. The plasmid contains the gene encoding resistance to the antibiotic kanamycin. Culturing the strain in the presence of the antibiotic should ensure that the plasmid is retained. However it is important that the plasmid be retained in the absence of the antibiotic selection as antibiotic selection is not possible *in vivo*.

10 A single colony of the strain K2 was inoculated into duplicate 10 ml cultures of L broth with and without kanamycin. The cultures were grown with shaking at 37°C for a total of 72 hours. Samples were taken at 30 and 48 hours post inoculation and serial dilutions plated onto L agar plates with and without kanamycin. The cultures were diluted 1/100 into Fresh L broth with and without  
15 kanamycin and cultured for a further 24 hours. Dilutions of the culture were again plated out onto L agar plates with and without kanamycin. Numbers of colony forming units (cfu) were recorded and are reported in Table 5.

### 3.4 Oral immunisation of mice with the strain K2.

20 The strain K2 was grown as described in 1.8 and used to challenge orally groups of 5 Balb/c mice (as previously described) with a dose range from  $10^4$  to  $10^{10}$  /dose. Deaths were recorded over 28 days and the LD<sub>50</sub>s calculated according to the method of Reed and Meunch (described in 2.3.2).

## 25 Results

### 4.1 Strain

The plasmid pLG339/*surA* was recovered from the strain K2 and digested with the two enzymes *SphI* and *Sall*. Separation of the resultant bands by agarose gel  
30 electrophoresis revealed the correct sized bands of 6.2 and 3 kb.



#### 4.2 Plasmid Stability

The presence of the plasmid pLG339/*surA* was investigated in the strain K2. The results show that in the absence of antibiotics the plasmid is retained by the bacteria. In these studies, at least 82% of the bacteria retain the plasmid when grown  
5 without antibiotics. This suggests that this plasmid should be maintained when the bacteria are used to infect mice.

#### 4.3 Complementation data

Groups of 5 Balb/c mice were orally challenged with various doses of the  
10 putative complemented strain K2. The oral LD<sub>50</sub> of the complemented strain K2 was calculated to be log<sub>10</sub>4.35 compared to that of log<sub>10</sub>4.17 for the parental strain C5.

Deaths of the mice within the group of mice challenged with log<sub>10</sub>8 bacteria of the three strains C5, BRD1115 and K2 are represented in Figure 5. Although the  
15 *surA* gene expressed from the plasmid appears to complement the defined mutation *in vivo*, the apparent delay in the time to death (when compared to the wild type parent strain) suggests the level of *surA* expression may be reduced in the strain K2.

## Tables

Table 1: Bacterial strains, plasmids and oligonucleotide primers used in this study

5	<u>Bacterial strains</u>	<u>Properties</u>	<u>Source or ref</u>
	<i>E.coli</i>		
	SY327	$\lambda$ pir lysogen	Miller V.L.(23)
	<i>S.typhimurium</i>		
10	LB5010	semi-rough	the inventor laboratory
	C5	wild type	C.Hormaeche, Cambridge, U.K.
	BRD441	TnphoA mutant, kan <sup>R</sup>	Miller I (21)
	BRD 1115		this study
	BRD 1126	amp <sup>R</sup>	Oxer M.D. (25)
15	BRD 1127	amp <sup>R</sup>	in press
	<u>Plasmids</u>		
	pBluescript <sup>0</sup> II SK+	amp <sup>R</sup>	Stratagene Ltd
	pGEM-T	amp <sup>R</sup>	Promega Corp.
20	pGP704	amp <sup>R</sup>	Miller V.L. (23)
	pGEM-T/212/213	amp <sup>R</sup>	this study
	pGEM-T/ $\Delta$ surA	amp <sup>R</sup>	this study
	pGP704/ $\Delta$ surA	amp <sup>R</sup>	this study
	pGEM-T/92/93	amp <sup>R</sup>	this study
25	pTETnir15	amp <sup>R</sup>	Oxer M.D. (25)
	pTET $\theta$ trA	amp <sup>R</sup>	in press
	<u>Oligo Primers</u>		
	MGR 92	TCGGCACGCAAGAAATGT	Kings College, London
30	MGR 93	AGACGACCAGTTCAATCG	" " "
	MGR 130	CGATGGGCTGAACTATTC	" " "
	MGR 135	TATGCAGCTTCGTTAGCG	" " "

Table 2: The oral and i.v. LD<sub>50</sub>'s of the three strains C5, BRD 441 and BRD 1115 were determined in BALB/c mice. Groups of 5 mice were immunised with doses ranging from log<sub>10</sub>4 to log<sub>10</sub>10 cfu of the strains BRD 441 and BRD 1115, and doses log<sub>10</sub>1 to log<sub>10</sub>5 of the strain C5. The results are presented in the following table.

40	Strain	oral LD <sub>50</sub> (log <sub>10</sub> cfu)	i.v.LD <sub>50</sub> (log <sub>10</sub> cfu)
	C5	4.16	<1.87
	BRD 441	8.62	2.46
	BRD1115	8.98	5.22

**Table 3:** The ability of the defined *surA* mutant strain to confer protection against homologous challenge with the wild type strain C5 was determined. Groups of 5 BALB/c mice were orally immunised with  $\log_{10}8$  organisms of the strain BRD1115 then challenged with  $\log_{10}4$  to  $\log_{10}10$  of the mouse virulent strain C5 either 4 or 10 weeks post inoculation. The new  $LD_{50}$  was then calculated and the results presented in the table below.

10	Immunising strain	oral $LD_{50}$ of C5		protection (no of $LD_{50}$ 's)
		4 weeks post immunisation	10 weeks post immunisation	
	BRD1115	8.58		~3800
	none	4.74		
15	BRD 1115		9.51	~4800
	none		4.68	

**Table 4:** Three groups of 10 mice were immunised with the strains BRD1115, BRD1126 and BRD1127 and then challenged 4 weeks post immunisation with 50  $LD_{50}$  doses of tetanus toxin subcutaneously. Deaths were noted over 4 days. The numbers of mice surviving the challenge are presented in the table below.

25 Strain	Survivors after challenge
BRD 1115	0/10
BRD 1126 ( <i>nirB</i> )	6/10
BRD 1127 ( <i>htrA</i> )	10/10

30

**Table 5:** The numbers of bacteria (cfu) present in the cultures of the complemented strain K2 following culture in L broth with and without the antibiotic kanamycin were calculated. The cultures were then plated onto L agar with and without kanamycin to show presence of the plasmid pLG339/*surA*. The results are presented as a total number and also the kanamycin resistant colonies as a percentage of the total bacteria present.

	Kanamycin Kanamycin		numbers of bacteria (cfu/ml)			
	in broth	in agar	30 hours (%)	48 hours (%)	72 hours (%)	
10	++	++	4.75x10 <sup>7</sup> (95%)	6x10 <sup>7</sup> (71%)	8.25x10 <sup>7</sup> (82.5%)	
	++	--	5 x10 <sup>7</sup>	8.5x10 <sup>7</sup>	10x10 <sup>7</sup>	
	--	++	5.25x10 <sup>7</sup> (124%)	9.5x10 <sup>7</sup> (111%)	6x10 <sup>7</sup> (89%)	
	--	--	4.25x10 <sup>7</sup>	8.5x10 <sup>7</sup>	6.75x10 <sup>7</sup>	

15

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**Claims**

1. A vaccine comprising a pharmaceutically acceptable carrier or diluent and a bacterium attenuated by a non-reverting mutation in a gene encoding a protein  
5 which promotes folding of extracytoplasmic proteins.
2. A vaccine according to claim 1 wherein the protein encoded by the mutant gene is a periplasmic protein.
- 10 3. A vaccine according to claim 1 or 2 wherein the protein encoded by the mutant gene promotes the folding of secreted proteins.
4. A vaccine according to claim 1, 2 or 3 wherein the protein encoded by the mutant gene is a peptidyl-prolyl cis-trans isomerase (PPIase).
- 15 5. A vaccine according to claim 4 wherein the PPIase is a member of the parvulin family of PPIases.
6. A vaccine according to any one of the preceding claims wherein the protein  
20 encoded by the mutant gene is SurA.
7. A vaccine according to any one of the preceding claims wherein the bacterium is further attenuated by a non-reverting mutation in a second gene.
- 25 8. A vaccine according to claim 7 wherein the second gene is an *aro* gene, a *pur* gene, the *htrA* gene, the *ompR* gene, the *galE* gene, the *cya* gene, the *crp* gene or the *phoP* gene.
9. A vaccine according to claim 8 wherein the *aro* gene is *aroA*, *aroC*, *aroD* or  
30 *aroE*.



10. A vaccine according to any one of the preceding claims wherein the mutation in the gene encoding a protein which promotes folding of extracytoplasmic proteins and/or the mutation in the second gene is a defined mutation.
- 5 11. A vaccine according to any one of the preceding claims wherein the bacterium has no uncharacterised mutations in the genome thereof.
12. A vaccine according to any one of the preceding claims wherein the bacterium is a bacterium that infects via the oral route.
- 10 13. A vaccine according to any one of the preceding claims wherein the bacterium is from the genera *Salmonella*, *Escherichia*, *Vibrio*, *Haemophilus*, *Neisseria*, *Yersinia*, *Bordetella* or *Brucella*.
- 15 14. A vaccine according to claim 13 wherein the bacterium is *Salmonella typhimurium*, *Salmonella typhi*, *Salmonella enteritidis*, *Salmonella choleraesuis*, *Salmonella dublin*, *Escherichia coli*, *Haemophilus influenzae*, *Neisseria gonorrhoeae*, *Yersinia enterocolitica*, *Bordetella pertussis* or *Brucella abortus*.
- 20 15. A vaccine according to any one of the preceding claims wherein the bacterium is genetically engineered to express an antigen from another organism.
16. A vaccine according to claim 15 wherein the antigen is fragment C of tetanus toxin.
- 25 17. A vaccine according to claim 15 or 16 wherein expression of the antigen is driven by the *nirB* promoter or the *htrA* promoter.
18. A bacterium as defined in any one of the preceding claims for use in a method of vaccinating a human or animal.
- 30

19. Use of a bacterium as defined in any one of the preceding claims for the manufacture of a medicament for vaccinating a human or animal.
20. A method of raising an immune response in a host, which method comprises  
5 administering to the host a bacterium attenuated by a non-reverting mutation in a gene encoding a protein which promotes folding of extracytoplasmic proteins.

10

15

Fig.1.

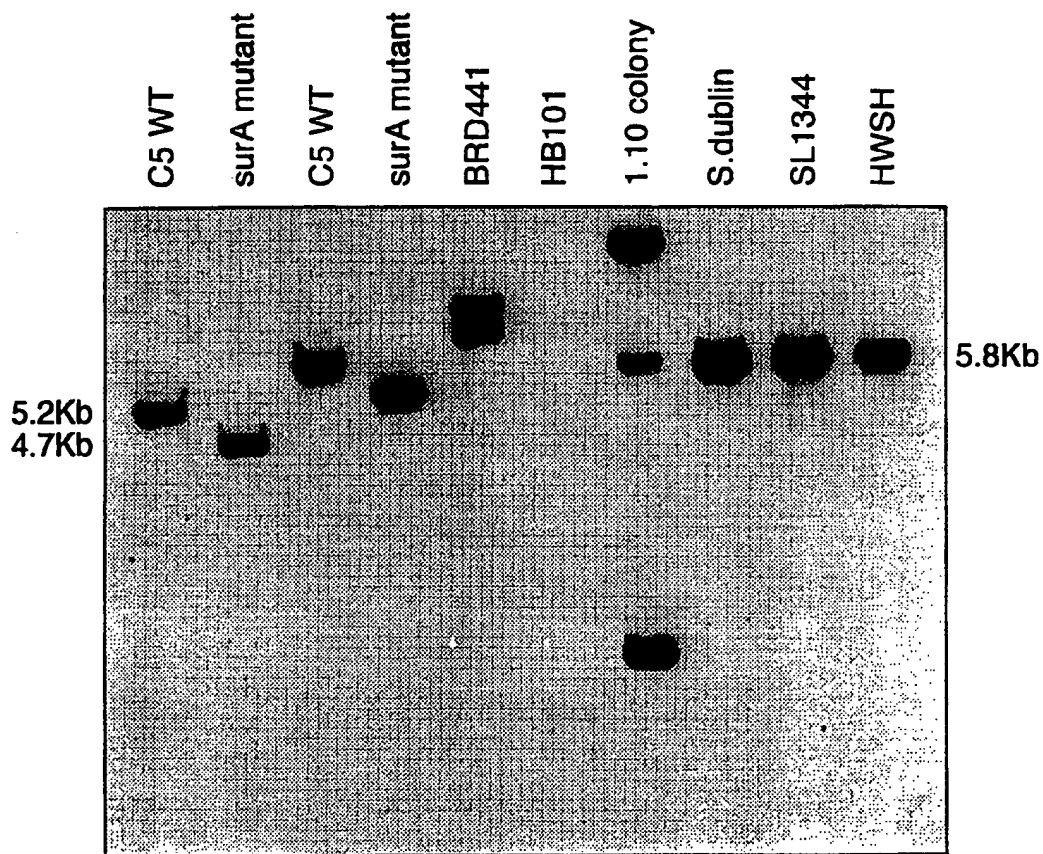
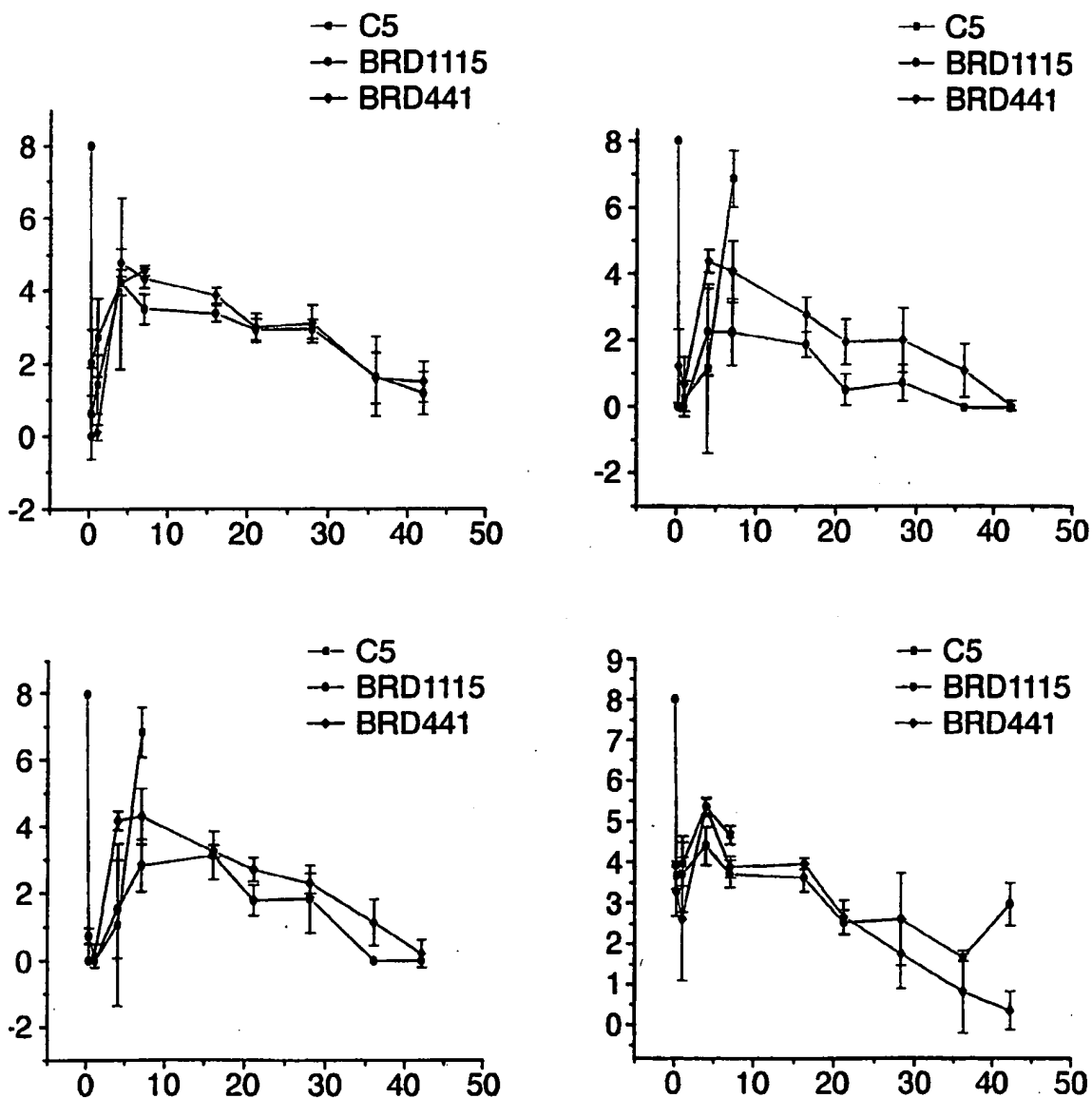


Fig.2.



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Fig.3.

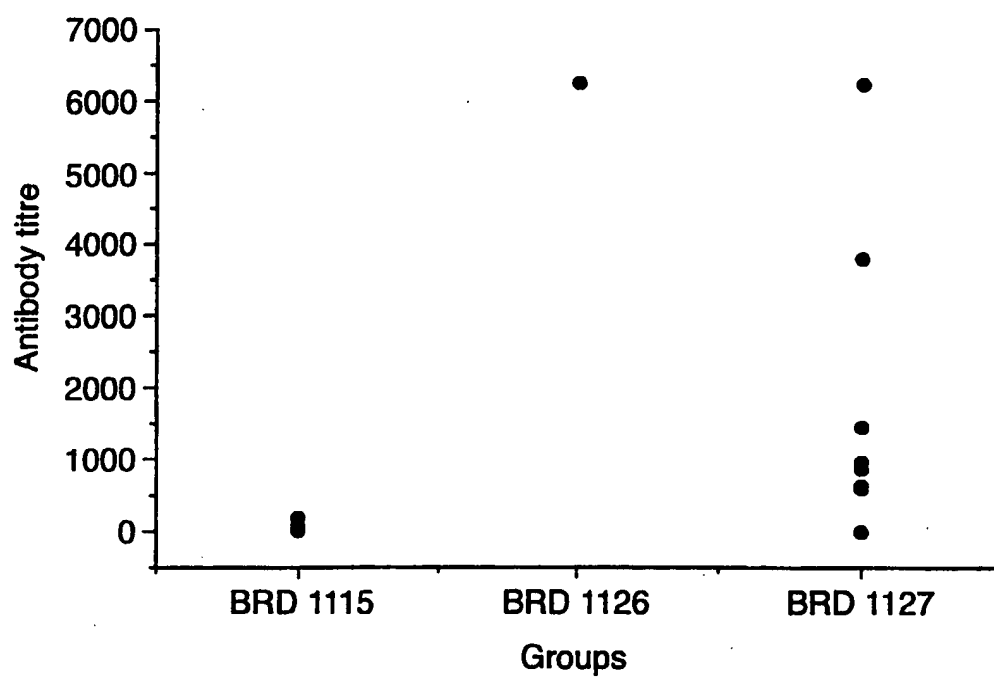


Fig.4.

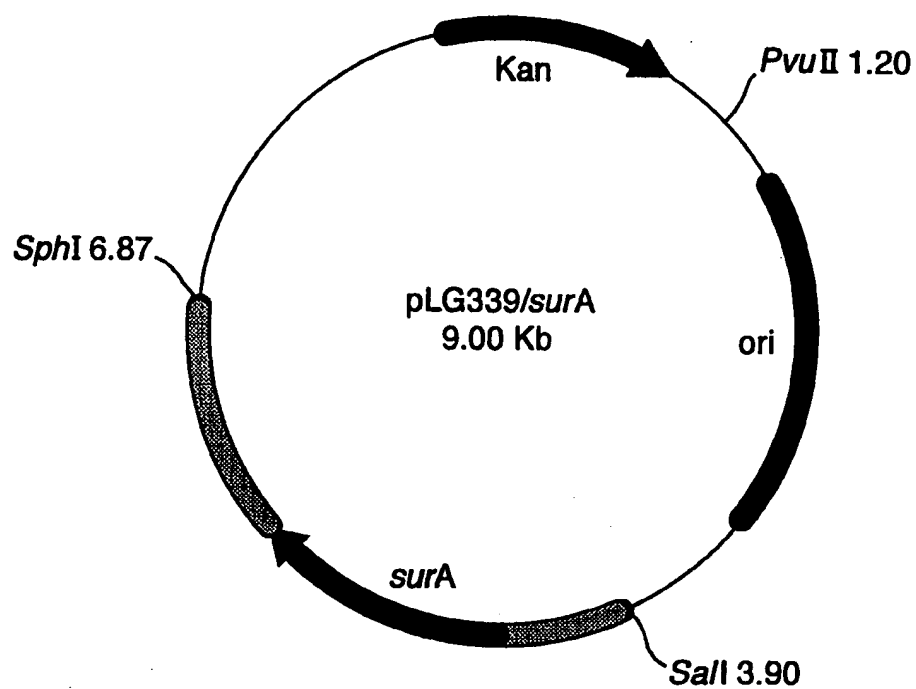
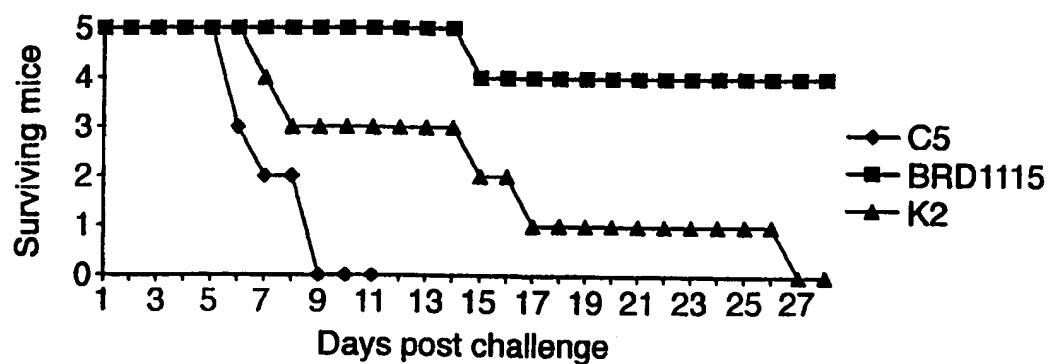


Fig.5.



**Sequence listing****(1) GENERAL INFORMATION:****(i) APPLICANT:**

(A) NAME: Medeva Europe Limited  
 (B) STREET: 10 St James's Street  
 (C) CITY: London  
 (D) STATE: not applicable  
 (E) COUNTRY: United Kingdom  
 (F) POSTAL CODE (ZIP): SW1A 1EF

(ii) TITLE OF INVENTION: VACCINES CONTAINING ATTENUATED BACTERIA

(iii) NUMBER OF SEQUENCES: 4

**(iv) COMPUTER READABLE FORM:**

(A) MEDIUM TYPE: Floppy disk  
 (B) COMPUTER: IBM PC compatible  
 (C) OPERATING SYSTEM: PC-DOS/MS-DOS  
 (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

**(2) INFORMATION FOR SEQ ID NO: 1:****(i) SEQUENCE CHARACTERISTICS:**

(A) LENGTH: 1287 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

**(vi) ORIGINAL SOURCE:**

(A) ORGANISM: Salmonella typhimurium

**(ix) FEATURE:**

(A) NAME/KEY: CDS  
 (B) LOCATION: 1..1281

**(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:**

ATG AAG AAC TGG AAA ACG CTG CTT CTC GGT ATC GCC ATG ATC GCG AAT	48
Met Lys Asn Trp Lys Thr Leu Leu Leu Gly Ile Ala Met Ile Ala Asn	
1 5 10 15	
ACC AGT TTC GCT GCC CCC CAG GTA GTC GAT AAA GTC GCA GCC GTC GTC	96
Thr Ser Phe Ala Ala Pro Gln Val Val Asp Lys Val Ala Ala Val Val	
20 25 30	
AAT AAT GGC GTC GTG CTG GAA AGC GAC GTT GAT GGC TTA ATG CAA TCA	144
Asn Asn Gly Val Val Leu Glu Ser Asp Val Asp Gly Leu Met Gln Ser	
35 40 45	

GTC AAA CTC AAC GCG GGT CAG GCA GGT CAG CAG CTT CCG GAC GAC GCC Val Lys Leu Asn Ala Gly Gln Ala Gly Gln Gln Leu Pro Asp Asp Ala 50 55 60	192
ACG CTG CGT CAC CAG ATC CTG GAA CGT TTG ATT ATG GAT CAA ATT ATC Thr Leu Arg His Gln Ile Leu Glu Arg Leu Ile Met Asp Gln Ile Ile 65 70 75 80	240
CTG CAG ATG GGT CAG AAG ATG GGG GTG AAG ATC ACG GAT GAG CAG TTG Leu Gln Met Gly Gln Lys Met Gly Val Lys Ile Thr Asp Glu Gln Leu 85 90 95	288
GAT CAG CCA TCA GCC AAC ATC GCC AAA CAA AAC AAT ATG ACG ATG GAT Asp Gln Pro Ser Ala Asn Ile Ala Lys Gln Asn Asn Met Thr Met Asp 100 105 110	336
CAG ATG CGC AGC CGT CTG GCT TAC GAT GGG CTG AAC TAT TCA ACC TAC Gln Met Arg Ser Arg Leu Ala Tyr Asp Gly Leu Asn Tyr Ser Thr Tyr 115 120 125	384
CGT AAC CAG ATT CGT AAA GAG ATG ATT ATC TCT GAA GTG CGC AAC AAT Arg Asn Gln Ile Arg Lys Glu Met Ile Ile Ser Glu Val Arg Asn Asn 130 135 140	432
GAG GTT CGT CGC CGT ATC ACC GTT TTG CCG CAA GAA GTT GAC GCG CTG Glu Val Arg Arg Arg Ile Thr Val Leu Pro Gln Glu Val Asp Ala Leu 145 150 155 160	480
GCA AAA CAG ATT GGC ACC CAA AAC GAT GCC AGC ACC GAG CTG AAC CTG Ala Lys Gln Ile Gly Thr Gln Asn Asp Ala Ser Thr Glu Leu Asn Leu 165 170 175	528
AGC CAT ATC CTG ATT GCT CTG CCG GAA AAC CCA ACC TCC GAG CAG GTT Ser His Ile Leu Ile Ala Leu Pro Glu Asn Pro Thr Ser Glu Gln Val 180 185 190	576
AAC GAC GCG CAG CGC CAG GCG GAA AGC ATT GTT GAA GAA GCG CGT AAC Asn Asp Ala Gln Arg Gln Ala Glu Ser Ile Val Glu Glu Ala Arg Asn 195 200 205	624
GGC GCA GAT TTC GGC AAA CTG GCG ATT ACC TAC TCT GCC GAC CAG CAG Gly Ala Asp Phe Gly Lys Leu Ala Ile Thr Tyr Ser Ala Asp Gln Gln 210 215 220	672
GCG CTA AAA GGC GGT CAG ATG GGC TGG GCG CGT ATC CAG GAG CTG CCG Ala Leu Lys Gly Gly Gln Met Gly Trp Gly Arg Ile Gln Glu Leu Pro 225 230 235 240	720
GGG ATT TTC GCC CAG GCG CTG AGC ACC GCG AAG AAA GGC GAC ATT GTC Gly Ile Phe Ala Gln Ala Leu Ser Thr Ala Lys Lys Gly Asp Ile Val 245 250 255	768
GGC CCG ATT CGC TCC GGC GTC GGC TTC CAC ATT CTG AAA GTA AAT GAC Gly Pro Ile Arg Ser Gly Val Gly Phe His Ile Leu Lys Val Asn Asp 260 265 270	816



CTG CGC GGT CAG AGC CAG AGT ATC TCC GTG ACC GAA GTT CAC GCT CGT Leu Arg Gly Gln Ser Gln Ser Ile Ser Val Thr Glu Val His Ala Arg 275 280 285	864
CAC ATT CTG CTT AAG CCG TCG CCG ATC ATG AAC GAT CAG CAG GCG CGC His Ile Leu Leu Lys Pro Ser Pro Ile Met Asn Asp Gln Gln Ala Arg 290 295 300	912
CTG AAG CTG GAA GAA ATC GCG GCT GAC ATT AAG AGT GGT AAA ACC ACC Leu Lys Leu Glu Glu Ile Ala Ala Asp Ile Lys Ser Gly Lys Thr Thr 305 310 315 320	960
TTT GCC GCT GCG GCG AAA GAG TAC TCT CAG GAC CCG GGC TCC GCT AAC Phe Ala Ala Ala Ala Lys Glu Tyr Ser Gln Asp Pro Gly Ser Ala Asn 325 330 335	1008
CAG GGC GGT GAT TTG GGT TGG GCT ACG CCA GAT ATT TTC GAC CCG GCG Gln Gly Gly Asp Leu Gly Trp Ala Thr Pro Asp Ile Phe Asp Pro Ala 340 345 350	1056
TTC CGC GAC GCG CTA ACG AAG CTG CAT AAA GGC CAA ATA AGC GCG CCG Phe Arg Asp Ala Leu Thr Lys Leu His Lys Gly Gln Ile Ser Ala Pro 355 360 365	1104
GTA CAC TCC TCT TTC GGC TGG CAT CTG ATC GAA TTG CTG GAT ACG CGT Val His Ser Ser Phe Gly Trp His Leu Ile Glu Leu Leu Asp Thr Arg 370 375 380	1152
AAG GTA GAC AAA ACC GAT GCG GCG CAG AAA GAT CGC GCT TAT CGT ATG Lys Val Asp Lys Thr Asp Ala Ala Gln Lys Asp Arg Ala Tyr Arg Met 385 390 395 400	1200
CTG ATG AAC CGT AAA TTC TCA GAA GAA GCG GCG ACC TGG ATG CAA GAA Leu Met Asn Arg Lys Phe Ser Glu Glu Ala Ala Thr Trp Met Gln Glu 405 410 415	1248
CAG CGC GCC ACT TAC GTT AAG ATT TTG AGT AAC TAATGA Gln Arg Ala Thr Tyr Val Lys Ile Leu Ser Asn 420 425	1287

## (2) INFORMATION FOR SEQ ID NO: 2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 427 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met	Lys	Asn	Trp	Lys	Thr	Leu	Leu	Leu	Gly	Ile	Ala	Met	Ile	Ala	Asn
1				5					10					15	
Thr	Ser	Phe	Ala	Ala	Pro	Gln	Val	Val	Asp	Lys	Val	Ala	Ala	Val	Val

20	25	30
Asn Asn Gly Val Val Leu Glu Ser Asp Val Asp Gly Leu Met Gln Ser		
35	40	45
Val Lys Leu Asn Ala Gly Gln Ala Gly Gln Gln Leu Pro Asp Asp Ala		
50	55	60
Thr Leu Arg His Gln Ile Leu Glu Arg Leu Ile Met Asp Gln Ile Ile		
65	70	75
Leu Gln Met Gly Gln Lys Met Gly Val Lys Ile Thr Asp Glu Gln Leu		
85	90	95
Asp Gln Pro Ser Ala Asn Ile Ala Lys Gln Asn Asn Met Thr Met Asp		
100	105	110
Gln Met Arg Ser Arg Leu Ala Tyr Asp Gly Leu Asn Tyr Ser Thr Tyr		
115	120	125
Arg Asn Gln Ile Arg Lys Glu Met Ile Ile Ser Glu Val Arg Asn Asn		
130	135	140
Glu Val Arg Arg Arg Ile Thr Val Leu Pro Gln Glu Val Asp Ala Leu		
145	150	155
Ala Lys Gln Ile Gly Thr Gln Asn Asp Ala Ser Thr Glu Leu Asn Leu		
165	170	175
Ser His Ile Leu Ile Ala Leu Pro Glu Asn Pro Thr Ser Glu Gln Val		
180	185	190
Asn Asp Ala Gln Arg Gln Ala Glu Ser Ile Val Glu Glu Ala Arg Asn		
195	200	205
Gly Ala Asp Phe Gly Lys Leu Ala Ile Thr Tyr Ser Ala Asp Gln Gln		
210	215	220
Ala Leu Lys Gly Gly Gln Met Gly Trp Gly Arg Ile Gln Glu Leu Pro		
225	230	235
Gly Ile Phe Ala Gln Ala Leu Ser Thr Ala Lys Lys Gly Asp Ile Val		
245	250	255
Gly Pro Ile Arg Ser Gly Val Gly Phe His Ile Leu Lys Val Asn Asp		
260	265	270
Leu Arg Gly Gln Ser Gln Ser Ile Ser Val Thr Glu Val His Ala Arg		
275	280	285
His Ile Leu Leu Lys Pro Ser Pro Ile Met Asn Asp Gln Gln Ala Arg		
290	295	300
Leu Lys Leu Glu Glu Ile Ala Ala Asp Ile Lys Ser Gly Lys Thr Thr		
305	310	315
		320

Phe Ala Ala Ala Lys Glu Tyr Ser Gln Asp Pro Gly Ser Ala Asn  
325 330 335

Gln Gly Gly Asp Leu Gly Trp Ala Thr Pro Asp Ile Phe Asp Pro Ala  
340 345 350

Phe Arg Asp Ala Leu Thr Lys Leu His Lys Gly Gln Ile Ser Ala Pro  
355 360 365

Val His Ser Ser Phe Gly Trp His Leu Ile Glu Leu Leu Asp Thr Arg  
370 375 380

Lys Val Asp Lys Thr Asp Ala Ala Gln Lys Asp Arg Ala Tyr Arg Met  
385 390 395 400

Leu Met Asn Arg Lys Phe Ser Glu Glu Ala Ala Thr Trp Met Gln Glu  
405 410 415

Gln Arg Ala Thr Tyr Val Lys Ile Leu Ser Asn  
420 425

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1287 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: E.coli

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION:1..1284

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

ATG AAG AAC TGG AAA ACG CTG CTT CTC GGT ATC GCC ATG ATC GCG AAT Met Lys Asn Trp Lys Thr Leu Leu Leu Gly Ile Ala Met Ile Ala Asn 430 435 440	48
ACC AGT TTC GCT GCC CCC CAG GTA GTC GAT AAA GTC GCA GCC GTC GTC Thr Ser Phe Ala Ala Pro Gln Val Val Asp Lys Val Ala Ala Val Val 445 450 455	96
AAT AAC GGC GTC GTG CTG GAA AGC GAC GTT GAT GGA TTA ATG CAG TCG Asn Asn Gly Val Val Leu Glu Ser Asp Val Asp Gly Leu Met Gln Ser 460 465 470 475	144
GTA AAA CTG AAC GCT GCT CAG GCA AGG CAG CAA CTT CCT GAT GAC GCG Val Lys Leu Asn Ala Ala Gln Ala Arg Gln Gln Leu Pro Asp Asp Ala	192

480	485	490	
ACG CTG CGC CAC CAA ATC ATG GAA CGT TTG ATC ATG GAT CAA ATC ATT Thr Leu Arg His Gln Ile Met Glu Arg Leu Ile Met Asp Gln Ile Ile 495 500 505			240
CTG CAG ATG GGG CAG AAA ATG GGA GTG AAA ATC TCC GAT GAG CAG CTG Leu Gln Met Gly Gln Lys Met Gly Val Lys Ile Ser Asp Glu Gln Leu 510 515 520			288
GAT CAG GCG ATT GCT AAC ATC GCG AAA CAG AAC AAC ATG ACG CTG GAT Asp Gln Ala Ile Ala Asn Ile Ala Lys Gln Asn Asn Met Thr Leu Asp 525 530 535			336
CAG ATG CGC AGC CGT CTG GCT TAC GAT GGA CTG AAC TAC AAC ACC TAT Gln Met Arg Ser Arg Leu Ala Tyr Asp Gly Leu Asn Tyr Asn Thr Tyr 540 545 550 555			384
CGT AAC CAG ATC CGC AAA GAG ATG ATT ATC TCT GAA GTG CGT AAC AAC Arg Asn Gln Ile Arg Lys Glu Met Ile Ile Ser Glu Val Arg Asn Asn 560 565 570			432
GAG GTG CGT CGT CGC ATC ACC ATC CTG CCG CAG GAA GTC GAA TCC CTG Glu Val Arg Arg Arg Ile Thr Ile Leu Pro Gln Glu Val Glu Ser Leu 575 580 585			480
GCG CAG CAG GTG GGT AAC CAA AAC GAC GCC AGC ACT GAG CTG AAC CTG Ala Gln Gln Val Gly Asn Gln Asn Asp Ala Ser Thr Glu Leu Asn Leu 590 595 600			528
AGC CAC ATC CTG ATC CCG CTG CCG GAA AAC CCG ACC TCT GAT CAG GTG Ser His Ile Leu Ile Pro Leu Pro Glu Asn Pro Thr Ser Asp Gln Val 605 610 615			576
AAC GAA GCG GAA AGC CAG GCG CGC GCC ATT GTC GAT CAG GCG CGT AAC Asn Glu Ala Glu Ser Gln Ala Arg Ala Ile Val Asp Gln Ala Arg Asn 620 625 630 635			624
GGC GCT GAT TTC GGT AAG CTG GCG ATT GCT CAT TCT GCC GAC CAG CAG Gly Ala Asp Phe Gly Lys Leu Ala Ile Ala His Ser Ala Asp Gln Gln 640 645 650			672
GCG CTG AAC GGC GGC CAG ATG GGC TGG GGC CGT ATT CAG GAG TTG CCC Ala Leu Asn Gly Gly Gln Met Gly Trp Gly Arg Ile Gln Glu Leu Pro 655 660 665			720
GGG ATC TTC GCC CAG GCA TTA AGC ACC GCG AAG AAA GGC GAC ATT GTT Gly Ile Phe Ala Gln Ala Leu Ser Thr Ala Lys Lys Gly Asp Ile Val 670 675 680			768
GGC CCG ATT CGT TCC GGC GTT GGC TTC CAT ATT CTG AAA GTT AAC GAC Gly Pro Ile Arg Ser Gly Val Gly Phe His Ile Leu Lys Val Asn Asp 685 690 695			816
CTG CGC GGC GAA AGC AAA AAT ATC TCG GTG ACC GAA GTT CAT GCT CGC			864

Leu Arg Gly Glu Ser Lys Asn Ile Ser Val Thr Glu Val His Ala Arg	
700 705 710 715	
CAT ATT CTG CTG AAA CCG TCG CCG ATC ATG ACT GAC GAA CAG GCC CGT	912
His Ile Leu Leu Lys Pro Ser Pro Ile Met Thr Asp Glu Gln Ala Arg	
720 725 730	
GTG AAA CTG GAA CAG ATT GCT GCT GAT ATC GAG AGT GGT AAA ACG ACT	960
Val Lys Leu Glu Gln Ile Ala Ala Asp Ile Glu Ser Gly Lys Thr Thr	
735 740 745	
TTT GCT GCC GCA ACG AAA GAG TTC TCT CAG GAT CCA GTC TCT GCT AAC	1008
Phe Ala Ala Ala Thr Lys Glu Phe Ser Gln Asp Pro Val Ser Ala Asn	
750 755 760	
CAG GGC GGC GAT CTC GGC TGG GCT ACA CCA GAT ATT TTC GAT CCG GCC	1056
Gln Gly Gly Asp Leu Gly Trp Ala Thr Pro Asp Ile Phe Asp Pro Ala	
765 770 775	
TTC CGT GAC GCC CTG ACT CGC CTG AAC AAA GGT CAA ATG AGT GCA CCG	1104
Phe Arg Asp Ala Leu Thr Arg Leu Asn Lys Gly Gln Met Ser Ala Pro	
780 785 790 795	
GTT CAC TCT TCA TTC GGC TGG CAT TTA ATC GAA CTG CTG GAT ACC CGT	1152
Val His Ser Ser Phe Gly Trp His Leu Ile Glu Leu Leu Asp Thr Arg	
800 805 810	
AAT GTC GAT AAA ACC GAC GCT GCG CAG AAA GAT CGT GCA TAC CGC ATG	1200
Asn Val Asp Lys Thr Asp Ala Ala Gln Lys Asp Arg Ala Tyr Arg Met	
815 820 825	
CTG ATG AAC CGT AAG TTC TCG GAA GAA GCA GCA AGC TGG ATG CAG GAA	1248
Leu Met Asn Arg Lys Phe Ser Glu Glu Ala Ala Ser Trp Met Gln Glu	
830 835 840	
CAA CGT GCC AGC GCC TAC GTT AAA ATC CTG AGC AAC TAA	1287
Gln Arg Ala Ser Ala Tyr Val Lys Ile Leu Ser Asn	
845 850 855	

## (2) INFORMATION FOR SEQ ID NO: 4:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 428 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Met Lys Asn Trp Lys Thr Leu Leu Leu Gly Ile Ala Met Ile Ala Asn  
 1 5 10 15

Thr Ser Phe Ala Ala Pro Gln Val Val Asp Lys Val Ala Ala Val Val  
 20 25 30

Asn Asn Gly Val Val Leu Glu Ser Asp Val Asp Gly Leu Met Gln Ser  
 35 40 45  
 Val Lys Leu Asn Ala Ala Gln Ala Arg Gln Gln Leu Pro Asp Asp Ala  
 50 55 60  
 Thr Leu Arg His Gln Ile Met Glu Arg Leu Ile Met Asp Gln Ile Ile  
 65 70 75 80  
 Leu Gln Met Gly Gln Lys Met Gly Val Lys Ile Ser Asp Glu Gln Leu  
 85 90 95  
 Asp Gln Ala Ile Ala Asn Ile Ala Lys Gln Asn Asn Met Thr Leu Asp  
 100 105 110  
 Gln Met Arg Ser Arg Leu Ala Tyr Asp Gly Leu Asn Tyr Asn Thr Tyr  
 115 120 125  
 Arg Asn Gln Ile Arg Lys Glu Met Ile Ile Ser Glu Val Arg Asn Asn  
 130 135 140  
 Glu Val Arg Arg Arg Ile Thr Ile Leu Pro Gln Glu Val Glu Ser Leu  
 145 150 155 160  
 Ala Gln Gln Val Gly Asn Gln Asn Asp Ala Ser Thr Glu Leu Asn Leu  
 165 170 175  
 Ser His Ile Leu Ile Pro Leu Pro Glu Asn Pro Thr Ser Asp Gln Val  
 180 185 190  
 Asn Glu Ala Glu Ser Gln Ala Arg Ala Ile Val Asp Gln Ala Arg Asn  
 195 200 205  
 Gly Ala Asp Phe Gly Lys Leu Ala Ile Ala His Ser Ala Asp Gln Gln  
 210 215 220  
 Ala Leu Asn Gly Gly Gln Met Gly Trp Gly Arg Ile Gln Glu Leu Pro  
 225 230 235 240  
 Gly Ile Phe Ala Gln Ala Leu Ser Thr Ala Lys Lys Gly Asp Ile Val  
 245 250 255  
 Gly Pro Ile Arg Ser Gly Val Gly Phe His Ile Leu Lys Val Asn Asp  
 260 265 270  
 Leu Arg Gly Glu Ser Lys Asn Ile Ser Val Thr Glu Val His Ala Arg  
 275 280 285  
 His Ile Leu Leu Lys Pro Ser Pro Ile Met Thr Asp Glu Gln Ala Arg  
 290 295 300  
 Val Lys Leu Glu Gln Ile Ala Ala Asp Ile Glu Ser Gly Lys Thr Thr  
 305 310 315 320  
 Phe Ala Ala Ala Thr Lys Glu Phe Ser Gln Asp Pro Val Ser Ala Asn

	325		330		335
Gln Gly Gly Asp Leu Gly Trp Ala Thr Pro Asp Ile Phe Asp Pro Ala					
	340		345		350
Phe Arg Asp Ala Leu Thr Arg Leu Asn Lys Gly Gln Met Ser Ala Pro					
	355		360		365
Val His Ser Ser Phe Gly Trp His Leu Ile Glu Leu Leu Asp Thr Arg					
	370		375		380
Asn Val Asp Lys Thr Asp Ala Ala Gln Lys Asp Arg Ala Tyr Arg Met					
	385		390		395
Leu Met Asn Arg Lys Phe Ser Glu Glu Ala Ala Ser Trp Met Gln Glu					
	405		410		415
Gln Arg Ala Ser Ala Tyr Val Lys Ile Leu Ser Asn					
	420		425		

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 98/03680

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 A61K39/02 A61K39/112 A61K39/108 A61K39/106 A61K39/102  
A61K39/095 A61K39/10 //C12N1/21,C12N1/36,C12R1/42

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LAZAR S W ET AL: "SurA assists the folding of Escherichia coli outer membrane proteins." JOURNAL OF BACTERIOLOGY, (1996 MAR) 178 (6) 1770-3. JOURNAL CODE: HH3. ISSN: 0021-9193., XP002099516 United States see the whole document	1-6
Y	---	7-20
	-/--	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

\* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

13 Apr11 1999

Date of mailing of the international search report

27/04/1999

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer

Covone, M



# INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 98/03680

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>SCHODEL F ET AL: "Salmonellae as oral vaccine carriers."  DEVELOPMENTS IN BIOLOGICAL STANDARDIZATION, (1995) 84 245-53. REF: 48  JOURNAL CODE: E7V. ISSN: 0301-5149., XP002099517  Switzerland  see page 246, paragraph 5  see page 247, paragraph 2-4  see page 250, paragraph 1</p>	7-20
X	<p>KLEEREBEZEM M ET AL: "Characterization of an Escherichia coli rotA mutant, affected in periplasmic peptidyl-prolyl cis/trans isomerase."  MOLECULAR MICROBIOLOGY, (1995 OCT) 18 (2) 313-20. JOURNAL CODE: MOM. ISSN: 0950-382X., XP002099518  ENGLAND: United Kingdom  see introduction  see page 317</p>	1-4
A	<p>WO 94 03615 A (MEDEVA HOLDINGS BV ;KHAN MOHAMMED ANJAM (GB); HORMAECHE CARLOS EST) 17 February 1994  see page 2, paragraph 2  see page 10, paragraph 2 - page 13, paragraph 2  see example 5  see claims 18-24</p>	1-20
A	<p>EP 0 400 958 A (WELLCOME FOUND ;ROYAL SOCIETY (GB); LISTER PREVENTIVE MED (GB); UN) 5 December 1990  see page 3, line 33-54  see page 4, line 27-36</p>	1-20
A	<p>LIU S L ET AL: "Rearrangements in the genome of the bacterium Salmonella typhi." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, (1995 FEB 14) 92 (4) 1018-22. JOURNAL CODE: PV3. ISSN: 0027-8424., XP002099519  United States  see page 1018, left-hand column, line 31-54  see page 1021, right-hand column, line 12 - page 1022, left-hand column, line 20</p>	1-20

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# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/GB 98/03680

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>RUDD K E ET AL: "A new family of peptidyl - prolyl isomerases." TRENDS IN BIOCHEMICAL SCIENCES, (1995 JAN) 20 (1) 12-4. JOURNAL CODE: WEF. ISSN: 0167-7640., XP002099520 ENGLAND: United Kingdom see the whole document ----</p>	1-20
P,X	<p>LAZAR S W ET AL: "Role of the Escherichia coli SurA protein in stationary-phase survival." JOURNAL OF BACTERIOLOGY, (1998 NOV) 180 (21) 5704-11. JOURNAL CODE: HH3. ISSN: 0021-9193., XP002099521 United States see page 5704, left-hand column, line 23 - right-hand column, line 20 see page 5709, left-hand column, line 1 - page 5710, right-hand column, line 39 -----</p>	1-6

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/GB 98/ 03680

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 20  
because they relate to subject matter not required to be searched by this Authority, namely:  
See FURTHER INFORMATION sheet PCT/ISA/210
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

**FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210**

Although claim 20 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/ composition.

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Claims Nos.: 20

Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/GB 98/03680

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9403615 A	17-02-1994	AT 174628 T	15-01-1999
		AU 4719393 A	03-03-1994
		CA 2141427 A	17-02-1994
		DE 69322645 D	28-01-1999
		EP 0652962 A	17-05-1995
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		DE 69022290 D	19-10-1995
		DK 400958 T	15-01-1996
		ES 2077028 T	16-11-1995
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## **Summary**

<b>Document</b>	<b>Pages</b>	<b>Printed</b>	<b>Missed</b>
<b>WO009949026</b>	<b>69</b>	<b>69</b>	<b>0</b>
<b>WO009937759</b>	<b>78</b>	<b>78</b>	<b>0</b>
<b>WO009929342</b>	<b>53</b>	<b>53</b>	<b>0</b>
<b>Total (3)</b>	<b>200</b>	<b>200</b>	<b>0</b>